IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

		ATTY.'s DOCKET: MUKAI=2
In re Application of:	)	Group Art Unit: 1651
Kazuhisa MUKAI et al.	)	Examiner: Lora E. Barnhart
Appln. No.: 10/523,920	)	Washington, D.C.
Date Filed: July 7, 2003	)	Confirmation No. 1923
For: PROCESS FOR PRODUCING...	)	August 26, 2010

DECLARRATION UNDER 37 CFR 1.132

Honorable Commissioner of Patents and Trademarks

Washington, D.C. 20231

Sir:

1. I, Tomoyuki NISHIMOTO, declare as follows:

2. I am a citizen of Japan, residing at 500-30 Meguro-cho, Okayama-shi, Okayama, Japan.

3. In 1985, I received a bachelor of Agriculture from Osaka Prefecture University, and in 1998 I received a doctorate of Agriculture from the above-identified university.

4. As shown in my curriculum vitae attached hereto as Attachment A, from 1990 to 2009, I researched in Hayashibara Biochemical Laboratories Inc. fundamental studies and industrial applications of carbohydrates and their related enzymes. Since 2009, I have been a director of Research

Center, Hayashibara Biochemical Laboratories, Inc.

5. I have read and thoroughly understood the present invention and the content of the United States Patent No. 5,137,723, titled "α-GLYCOSYL-L-ASCORBIC ACID, AND ITS PREPARATION AND USES" applied for by "Kabushiki Kaisha Hayashibara Seibutsu Kagaku Kenkyujo" (Hayashibara Biochemical Laboratories, Inc.), cited in an official action in the procedure of the present invention.

6. In the declaration dated November 28, 2008, I demonstrated on the basis of the experimental results that the α-isomaltosyl glucosaccharide-forming enzyme (abbreviated as "IMG", hereinafter) according to the present invention is significantly different from rat intestine α-glucosidase (RIAGase) described in the above-identified patent in the productivity of 2-O-α-D-glucopyranosyl-L-ascorbic acid (AA2G), 5-O-α-D-glucopyranosyl-L-ascorbic acid (AA5G), and 6-O-α-D-glucopyranosyl-L-ascorbic acid (AA6G). The experiment was conducted using "PINEDEX® #1", a partial starch hydrolyzate having a dextrose equivalent (DE) of about 8±1, as a substrate.

7. This time, I conducted the following experiment using three kinds of partial starch hydrolyzate with a different DE in the range of about 2 to 9 to demonstrate that my conclusion in the previous declaration remains same at least when liquefied starch with a DE of less than 10 is used as a substrate.

8. <u>Formation of AA2G by the enzymes</u>

<u>Experiment 8-1: Materials</u>

Two kinds of partial starch hydrolyzates, "PINEDEX® #1" (DE 8±1) and "PINEDEX® #100" (DE 2-5), were purchased from Matsutani Chemical Industries Co., Ltd., Hyogo, Japan. (DEs of "PINEDEX® #1" and "PINEDEX® #100" are shown in the pamphlet of "PINEDEX®" distributed by Matsutani Chemical Industries Co., Ltd., Hyogo, Japan, copy attached.) A liquefied corn starch solution (DE 4.1) was prepared in our laboratory according to the following method. A corn starch was prepared into about 20% starch suspension, admixed with calcium carbonate to give a final concentration of 0.1%, adjusted to pH 6.5, and admixed with 0.3%/g-starch of "THERMAMYL® 60 L", an α-amylase commercialized by Novozymes Japan, Chiba, Japan, and then heated at 95°C for 15 minutes. After autoclaving at 120°C for 20 minutes, the reaction mixture was cooled to 53°C to obtain a liquefied corn starch solution. The DE of the liquefied corn starch was determined to be 4.1 by conventional Lane-Eynon method.

IMG from *Arthrobacter globiformis* A19 (FERM BP-7590) and RIAGase were prepared and partially purified, respectively, according to the method in Experiments 7 and 8 described in my previous declaration on November 28, 2008.

Experiment 8-2: Enzyme reaction

Three kinds of aqueous solution containing 5% (w/v) of L-ascorbic acid and 5% (w/v) of "PINEDEX® #1", "PINEDEX® #100", or the above liquefied corn starch, as a glucosyl donor, adjusted to pH 5.3 were used as substrates for IMG and RIAGase. To each substrate solution, 20 units/g-glucosyl donor of the partially purified IMG or 20 units/g-glucosyl donor of the partially purified RIAGase was added and subjected to an enzyme reaction at 50°C for 24 hours. After the reaction, the reaction mixture was boiled for 10 min to inactivate IMG or RIAGase.

Then, each reaction mixture, obtained by allowing IMG or RIAGase to act on the substrate, was admixed with 40 units/g-glucosyl donor of

glucoamylase commercialized by Seikagaku Corporation, Tokyo, Japan, to hydrolyze the remaining partial starch hydrolyzate into glucose and subjected to an enzyme reaction at 40°C for 17 hours. After the reaction, each reaction mixture was boiled for 10 min to inactivate glucoamylase. The resulting each solution was subjected to high performance liquid chromatography (HPLC) for determining the contents of AA2G, AA5G, and AA6G. HPLC analysis was carried out according to the method described in Experiments 5 and 7 of the specification of the present invention.

9. Experimental results

The results of Experiment 8 are summarized in Table 1.

Table 1

Enzyme	Glucosyl donor	Dextrose Equivalent (DE)	Content in reaction mixture (%, on a dry solid basis)		
			AA2G	AA5G	AA6G
IMG*	PINEDEX® #1**	8±1	25.1	ND***	ND***
	PINEDEX® #100**	2-5	25.6	ND***	ND***
	Liquefied corn starch	4.1	26.2	ND***	ND***
RIAGase	PINEDEX® #1**	8±1	14.8	1.9	0.3
	PINEDEX® #100**	2-5	14.9	2.0	0.3
	Liquefied corn starch	4.1	15.1	2.1	0.3

*: IMG from *Arthrobacter globiformis* A19 (FERM BP-7590)

**: Partial starch hydrolyzate commercialized by Matsutani Chemical Industries Co., Ltd., Hyogo, Japan

***: Not detected

As evident from the results in Table 1, AA2G content in the reaction mixtures reached about 25.1 to 26.2%, on a dry solid basis, when IMG from

Arthrobacter globiformis A19 (FERM BP-7590) was allowed to act on "PINEDEX® #1" with a DE of 8±1, "PINEDEX® #100" with a DE of 2 to 5, and liquefied corn starch with a DE of 4.1, respectively. In addition, AA5G and AA6G, as by-products, were not detected in the reaction mixtures.

On the contrary to this, when RIAGase was allowed to act on the substrates with a DE of 8±1, 2 to 5, and 4.1, the AA2G contents in the reaction mixture were 14.8 to 15.1%, on a dry solid basis. Further, RIAGase produced AA5G and AA6G, as by-products, in amounts of about 2.0% and 0.3%, respectively.

10. Conclusion:

The above experimental results indicate that IMG from *Arthrobacter globiformis* A19 (FERM BP-7590) is superior to RIAGase in the productivity of AA2G when at least the DE of the substrate, i.e. liquefied starch, is in the range of about 2 to 9. Further, the above results indicate that IMG from *Arthrobacter globiformis* A19 (FERM BP-7590) is more suitable than RIAGase for producing AA2G at least the DE of the substrate, i.e. liquefied starch, is in the range of about 2 to 9, since IMG does not produce AA5G and AA6G as by products, while RIAGase does. It is believed that IMG from *Arthrobacter globiformis* A19 (FERM BP-7590) is a significantly different enzyme from RIAGase in the productivities of both AA2G and by products such as AA5G and AA6G.

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001,

and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Tomoyuki Nishimoto

NAME: Tomoyuki NISHIMOTO

26th day of August, 2010

DATE: 26th day of August, 2010

Attachment A

CURRICULUM VITAE

Name: Tomoyuki NISHIMOTO

Affiliation: Hayashibara Biochemical Laboratories, Inc.,
675-1, Fujisaki, Okayama-shi, Okayama,
Japan 702-8006
Tel: +81 86 276 8670

Date of Birth: December 26, 1961

Education:
Granted and received a bachelor from Osaka Prefecture University, Agricultural Department in 1985.
Granted and received a master's degree from Osaka Prefecture University, Agricultural Department in 1987.
Received a doctorate of Agriculture at Osaka Prefecture University in 1998.

Brief Chronology of Employment:

1987 (April)	Researcher, Hayashibara Co., Ltd.
1987 (July)	Researcher, Toyama Medical and Pharmaceutical University, under the employment of Hayashibara Biochemical Laboratories, Inc.
1990	Researcher, Amase Institute, Research Center, Hayashibara Biochemical Laboratories, Inc.
2002	Senior Scientist, Amase Institute, Research Center, Hayashibara Biochemical Laboratories, Inc.
2004	Chief Scientist, Amase Institute, Research Center, Hayashibara Biochemical Laboratories, Inc.
2006	Chief Scientist, Glycoscience Institute, Research Center, Hayashibara Biochemical Laboratories, Inc.
2009 (April)	Director, Glycoscience Institute, Research Center, Hayashibara Biochemical Laboratories, Inc.

Public Employment:

2006 (September)-	Member of Editorial Board of Journal Applied Glycoscience
2010 (April)-	Affiliate associate professor of Hiroshima University
2010 (April)-	Part-time assistant professor of Kurashiki Sakuyo University

Attachment B

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